**Biotransformation and metabolic profile of catalpol with human
intestinal microflora by Ultra-performance liquid chromatography
coupled with quadrupole time-of-flight mass spectrometry**

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Abstract

Traditional Chinese medicine (TCM) has been used in clinical practice for thousands of years. Catalpol, an iridoid glucoside, abundantly found in the root of the common used herb medicine *Rehmannia glutinosa* Libosch, has been reported to show various biological effects and pharmacological activities. After oral administration, the active ingredient might have interactions with the intestinal bacteria, which could help unravel how the medicine was processed *in vivo*. In this work, different pure bacteria from healthy human feces were isolated and used to bioconvert catalpol. Ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF/MS) technique combined with Metabolynx^{TM} software was applied to analyze catalpol metabolites. Compared with blank samples, parent compound (M0) and four metabolites (M1-M4) were detected and tentatively identified based on the characteristics of their protonated ions. The metabolites were likely to be: catalpol aglycone (M1), acetylated catalpol (M2), dimethylated and hydroxylated catalpol aglycone (M3), nitrogen-containing catalpol aglycone (M4). M1 and M4 were generated in the majority of the samples like *Bacteroides sp.* 45. M3 was obtained in several bacterial samples like *Enterococcus sp.* 8-2 and M2 was detected only in the sample of *Enterococcus sp.* 43-1. To our knowledge, the metabolic routes and metabolites of catalpol produced by human intestinal bacteria were all firstly reported.

Keywords: Catalpol, Human intestinal bacteria, UPLC-Q-TOF/MS, Metabolites

1. Introduction

Rehmannia (known as Dihuang) has long been used extensively as a traditional health food in East Asia and displays numerous biological activity. Catalpol, an iridoid glucoside contained richly in the roots of the small flowering plant species *Rehmannia glutinosa* Libosch, has been shown to have antioxidation, anti-inflammation, anti-apoptosis, anti-brain ischemia, anti-senile dementia, and other neuroprotective properties and plays a role in neuroprotection against hypoxic/ischemic injury, Alzheimer disease and Parkinson's disease both in vivo and in vitro models. [1-3]. It has protective effects against H₂O₂-induced oxidative damage in astrocytes [4] and may possess therapeutic potential against LPS-induced acute systemic inflammation by attenuating NF-κB activation and protecting mitochondrial function in cerebral cortex and hippocampus [2,5]. Catalpol has been reported to rescue neurons from kinds of damage *in vitro* and *in vivo*. It improved memory and protected the forebrain neurons from neurodegeneration through increasing brain derived neurotrophic factor (neuronotrophic) (BDNF) expression [6], protected primary cultured cortical neurons induced by Abeta(1-42) through a mitochondrial-dependent caspase pathway [7]. Studies also have shown that catalpol was a potential agent for the prevention of neurons apoptosis by regulating NO and iNOS increase in ERK-mediated neurodegenerative disorders [8].

The microbial community in the gut contains three domains of life: bacteria, archaea and eukarya (fungi, yeasts and protozoa), with the largest community residing

in the colon. Indeed, the human colon harbors a highly complex microbial ecosystem of about 200 g living cells, at concentrations of 10^{12} microorganisms per gram gut content, the highest recorded for any microbial habitat. Together, all 6.5 billion humans on earth represent a gut reservoir of 10^{23} - 10^{24} microbial cells, which is just five orders of magnitude less than the world's oceans (10^{29} cells) [9-10]. Therefore, the human gut constitutes a substantial habitat in our biosphere. The endogenous gastrointestinal microbial flora plays a fundamentally important role in human health and disease, yet this ecosystem remains incompletely characterized and its diversity poorly defined. The intestinal microbiota can therefore be regarded as a separate organ within the human host, which is capable of even more conversions than the human liver.

Most herbal medicines are administered orally and their components are inevitably brought into contact with intestinal microflora in the alimentary tract. Most components are metabolized by the intestinal bacteria before absorption from the gastrointestinal tract [11]. Intensive metabolism results in often low circulating levels of the original products, with the consequence that final health effects of botanicals are often related to specific active metabolites which are produced in the body rather than being related to the product's original composition. Understanding how such metabolic processes contribute to the *in situ* exposure is therefore crucial for the proper interpretation of biological responses [12].

However, little is known about the roles of human intestinal bacteria in the metabolic processes and biological activities of catalopls now. The aim of this work

was to isolate different pure bacteria from human feces and investigate the capability of human gut bacteria to biotransform catalopl. To further clarify the metabolic profile of catalopl by the isolated human intestinal bacteria, a rapid and sensitive ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) with automated data analysis (MetaboLynx) was established to identify its metabolites [13]. The results probably provided helpful information for further *in vivo* metabolism and action mechanism research on catalopl.

2. Experimental

2.1 Materials

Catalopl standard substance (purity, >98%) was purchased from Shanghai Winherb Medical S &T Development Co. Ltd (Shanghai, China). The formic acid was obtained from Merck KGaA (Darmstadt, Germany). The HPLC-grade acetonitrile was purchased from TEDIA Company Inc. (Fairfield, USA). The distilled water was purified by an EPED super purification system (Nanjing, China). Other reagents were of analytical grade. AnaeroPack Rectangular Jars were purchased from Mitsubishi Gas Chemical Company INC (Japan). General anaerobic medium (GAM) was purchased from Shanghai Kayon Biological Technology Co. Ltd. (Shanghai, China).

2.2 Organism

The human fecal specimens were prepared according to previous methods [14].

Briefly, 4 g of human fresh feces from a healthy Chinese man (28-year-old, no smoking, no current medication, no use of antibiotics, and fasting before sampling) were collected in a plastic cup. The sample was then suspended in 20 ml sterile physiological saline and homogenized adequately by a vortex-mixer. The fecal mixture was centrifuged for 10 min and the supernatant was then filtered with gauze. The suspension was used as a metabolic intestinal microflora fraction.

The bacterial mixture was diluted serially in aquae sterilisata and each of the dilutions was spread on the GAM agar plates which were incubated under anaerobic condition in anaerobic jars for 48 h at 37 °C. At last, about one hundred different types of bacterial colonies developed on plates were observed [15]. Each bacterium picked up from GAM agar plate was inoculated into 1.0 mL GAM broth and anaerobically incubated at 37 °C for 36 h.

2.3 Preparation of catalopl sample for analysis

The standard solution was prepared by dissolving accurately weighed catalopl in methanol to give a final concentration of 3.0 mg/mL. 0.1 mM catalopl was inoculated into 0.9 mL of GAM broth with 0.1 mL precultured bacteria and anaerobically incubated at 37 °C for 36 h. The sample was extracted twice with acetone, and the acetone layer dried under vacuum and dissolved with 0.3 mL methanol. The water layer was extracted twice with butanol, and the butanol layer dried under vacuum and dissolved with 0.3 mL methanol, combined with the acetone layer. The mixture was centrifuged at $12,000 \times g$ for 10 min before being injected for. UPLC/MS analysis.

2.4 UPLC-MS spectrometry

Catalopl and its metabolites were separated and identified on a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) fitted with a Synchronis C 18 column (100 mm $\times$ 2.1 mm i.d., 1.7 μ m; Thermo, USA). The chromatographic conditions were optimized to give good separation of the target compounds and avoid the interferences. The column temperature was maintained at 35 $^{\circ}$ C. A gradient elution system was used with mobile phase A (0.1 % formic acid in distilled water) and mobile phase B (acetonitrile) at a total flow rate of 0.4 ml/min. The gradient program was started at 95 % mobile phase A and 5 % mobile phase B, changed linearly to 10 % mobile phase A and 90 % mobile phase B for 11 min, and was held for 1 min. The gradient was recycled back to 5 % in 1 min for the next run. The injection volume was 5 μ L. The retention time of catalopl was 0.91 min.

For analysis, a Waters ACQUITY Synapt mass spectrometer (Waters Corp., Manchester, UK) connected to the UPLC system via an electrospray ionization (ESI) interface was used. ESI in both positive and negative ion modes were attempted in the method development. Since the metabolites were more abundant in positive ion mode. The characterization of the metabolites was based on their ion fragmentation in ESI⁺ mode. In the full scan mass spectra, the protonated molecular ions [M+H]⁺ were more stable and exhibited higher abundance than [M+Na]⁺, so the [M+H]⁺ was chosen as the precursor ion for MS/MS fragmentation analysis.

Product ion mass spectra were obtained at different desolvation temperature,

source temperature, desolvation gas flow and capillary voltage to select maximum sensitivity for most constituents. The temperature of the ion source was maintained at 120 °C and desolvation temperature was 350 °C. The gas (N₂) flows of cone and desolvation were 50 and 600 L/h, respectively. The capillary voltage used for the analysis of catalopl and its metabolites was 3.0 kV. All data collected in centroid mode were acquired using Masslynx^{NT} 4.1 software (Waters Corp., Milford, MA USA).

3. Results and discussion

3.1 Metabolic profile of catalopl by the different human intestinal microflora

A full overview of the catalopl metabolic profile was obtained after optimizing the UPLC and MS conditions. Using the positive ion electrospray tandem mass spectrometry and the Metabolynx^{TM} program, catalopl (M0) and its four metabolites (Table 1) were detected in different bacteria samples compared with blank samples. These metabolites were likely to be: catalopl aglycone (M1), acetylated catalopl (M2), dimethylated and hydroxylated catalpol aglycone (M3) and nitrogen-containing catalpol aglycone (M4). M1 and M4 were obtained in the majority of samples of the human intestinal bacteria isolated from the feces in this study. However, M3 was generated in several bacterial samples like *Enterococcus sp.* 8-2 and M2 was detected only in the sample of *Enterococcus sp.* 43-1. The extracted ion chromatograms (XICs) from the total UPLC/MS chromatograms were shown in Fig. 1.

3.2 Identification of catalopl metabolites

3.2.1 Parent compound

Catalopl (M0) was identified by comparing the UPLC retention time (0.91 min), accurate MS at m/z 363.1292 $[M+H]^+$ and MS/MS spectra with the authentic standard (Fig. 2a). As illustrated in the fragmentation pathway (Fig. 3a), the precursor ion $[M+H]^+$ m/z 363.1292 afforded the catalopl aglycone product ion at m/z 201.3302. The product ions at m/z 183.1218, 165.1009 and 147.2001 were formed from m/z 201.3302 by loss of H_2O , $2H_2O$ and $3H_2O$, respectively. The other characteristic fragments were m/z 137.0019 and 109.0021 corresponding to the loss of CO and 2CO from m/z 165.1009.

3.2.2 Metabolite M1

The retention time and fragment ion of metabolite M1 were at 2.95 min and m/z 201.3302, respectively. Fragment ion at m/z 201.3302 was generated from M0 m/z 363.1292 by loss of the glucuronide group. According to the MS/MS spectrum and the fragmentation regularity (Fig. 2b and Fig. 3b), the other fragment ions of M1 were also yielded in the mass spectrum of M0. Thus, M1 was identified as the aglycone of catalopl.

3.2.3 Metabolite M2

Metabolite M2 showed an UPLC profile with a retention time at 6.41 min (Fig. 2c). The fragment ion of M2 was at m/z 243.4210 which was 42 Da higher than M1

and it was also fragmented to give m/z 201.3302, 183.1218, 165.1009, 147.2001, 137.0019, 109.0021 (Fig. 3c). Therefore, M2 was identified as acetylated catalpol aglycone.

3.2.4 Metabolite M3

The fragment ion of M3 (retention time 4.35 min) was at m/z 247.4360, which was 46 Da higher than M1 (Fig. 2d). Fragment ion at m/z 229.1017 was obtained from M3 by loss of H_2O . Then, the m/z 229.1017 lost CH_2O and $2CH_2O$ to give m/z 199.0261 and 169.3016, respectively (Fig. 3d). So, M3 was identified as dimethylated and hydroxylated catalpol aglycone.

3.2.5 Metabolite M4

Metabolite M4 was detected as deprotonated molecular ion $[M+H]^+$ at m/z 200.2514 with retention time of 3.42 min (Fig. 2e), which was 1 Da lower than that of M1. The product ions at m/z 182.3021 and 164.0381 were formed by the elimination of H_2O and $2H_2O$ from m/z 200.0514, respectively (Fig. 3e). It was reported that morroniside, geniposide and gardenoside were transformed to new nitrogen-containing compounds, mor-1 and mor-2, genipinine and gardenine, respectively, along with the known aglycones [16]. In common with these compounds, catalpol is also an iridoid glycoside. Based on the above analysis, the similar metabolic pathway was possible for catalpol and M4 was proposed to be a nitrogen-containing compound.

3.3. Proposed metabolic pathways of catalopl by different human intestinal bacteria

According to our experimental results, the metabolic pathways of catalopl by human intestinal bacteria were proposed as shown in Fig. 4. After incubation with the isolated bacteria, M1 in the majority sample was identified as catalopl deglycosylated metabolite. Some other bacteria like *Enterococcus sp.* 43-1 showed moderate activities for the aglycone. From these results, we could deduce that catalopl was firstly metabolized to be catalopl aglycone by β -D-glucosidase from these bacteria, thereafter catalopl aglycone was metabolized to be acetylated and dimethylated and hydroxylated catalopl. It was reported that morroniside, a bioactive iridoid glycoside, could be firstly hydrolyzed to its aglycone. After the cleavage of B-ring of the aglycone, two steps of nucleophilic addition reaction took place to produce an N-heterocycle derivative, and this derivative further dehydrated to form mor-1; and the product from the cleavage of A- and B-ring of the aglycone would produce mor-2 via a step of benzoin condensation and two steps of nucleophilic addition reaction [17]. Due to the similar chemical structure with morroniside, the metabolic pathway was possible for catalopl. Thus, we proposed nitrogen-containing catalopl aglycone was produced after the aglycone being released.

There are 100 trillion microbes in the human gastrointestinal tract with numbers increasing distally. These microbiota secrete a diverse array of enzymes (primarily for carbohydrate and protein fermentation) giving them substantial metabolic potential which can have major implications for drug stability [18]. The set of the colonic

microbial community genes that code for metabolic enzymes is highly diverse and redundant to ensure survival under fluctuating nutritional conditions [12]. The hydrolytic function is essential for providing bacteria with carbon and energy from the released sugars and/or oligosaccharides. The activities of the various hydrolases are mainly associated with the bacterial fraction in fecal samples [19,20]. While, methylation can greatly enhance the medicine metabolic stability [21] and improve its lipophilicity than the prototype drug.

The naturally occurring iridoid catalpol is a Taq DNA polymerase inhibitor. However, its poor lipophilicity might account for the lack of biological activity against human solid tumor cell lines. The traditional prodrug approach by means of peracetylation of the free hydroxyl groups led to a compound, which showed a marginal growth inhibition against the most sensitive cell line A2780 (ovarian cancer) [22]. In this study, acetylated product of catalpol also could be produced by human intestinal bacteria. Compared with the traditional prodrug approach, biotransformation way might have its special advantages.

4. Conclusion

In this paper, a method using ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) with automated data analysis (MetaboLynx^{TM}) was established for search and identification of catalpol metabolites produced by human intestinal bacteria *in vitro*. Four catalpol metabolites were tentatively identified and the metabolic pathways were

firstly proposed, respectively. To our knowledge, catalpol, a poor lipophilicity and the water-soluble component, has a low bioavailability. But biotransformation might improve the bioavailability [15]. Investigation of the metabolism of catalpol by intestinal bacteria *in vitro* could help understand the metabolism of the component after oral administration and would be useful for tracing the therapeutic effects of medications for certain disease. However, further studies were still needed to identify the enzyme system which contributed to the metabolism and reaction mechanisms by the intestinal bacteria.

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Figure captions

Fig. 1. UPLC/MS chromatograms of catalpol and its metabolites. (a) GAM with standard solution of catalpol sample, (b) *Bacteroides* sp. 45 sample, (c) *Enterococcus* sp. 8-2 sample, (d) *Enterococcus* sp. 43-1 sample.

Fig. 2. UPLC-MS/MS spectra. (a) M0 (m/z 363), (b) M1 (m/z 201), (c) M2 (m/z 243),
(d) M3 (m/z 247), (e) M4 (m/z 200).

Fig. 3. Proposed fragmentation pathways of M0-M4.

Fig. 4. Proposed metabolic pathways of catalpol by human intestinal bacteria.

Figure 1

Fig. 1

Figure 2

(b)

(d)

Fig. 2

Figure 3

(a)

(b)

(c)

(d)

(e)

Fig. 3

Figure 4

Fig. 4

Table 1

Catalpol and its metabolites identified in different human intestinal bacterial samples using UPLC-MS/MS

Peak	<i>t</i> _R (min)	[M+H] ⁺ (m/z)	MS ² fragment ions (m/z)	Identification	Formula	Error (ppm)
M0	0.91	363.1292	363, 201, 183, 165, 147, 137, 109	Catalpol	C ₁₅ H ₂₂ O ₁₀	-2.0
M1	2.95	201.3302	201, 183, 165, 147, 137, 109	Catalpol aglycone	C ₉ H ₁₂ O ₅	1.3
M2	6.41	243.4210	243, 201, 183, 165, 147, 137, 109	Acetylated catalpol	C ₁₁ H ₁₄ O ₆	0.9
M3	4.35	247.4360	247, 229, 199, 169	Dimethylated and hydroxylated catalpol aglycone	C ₁₁ H ₁₈ O ₆	-1.4
M4	3.42	200.2514	200, 182, 164	Nitrogen-containing catalpol aglycone	C ₉ H ₁₃ O ₄ N	1.0

*t*_R, retention time; [M+H]⁺, positive charged molecular ion.